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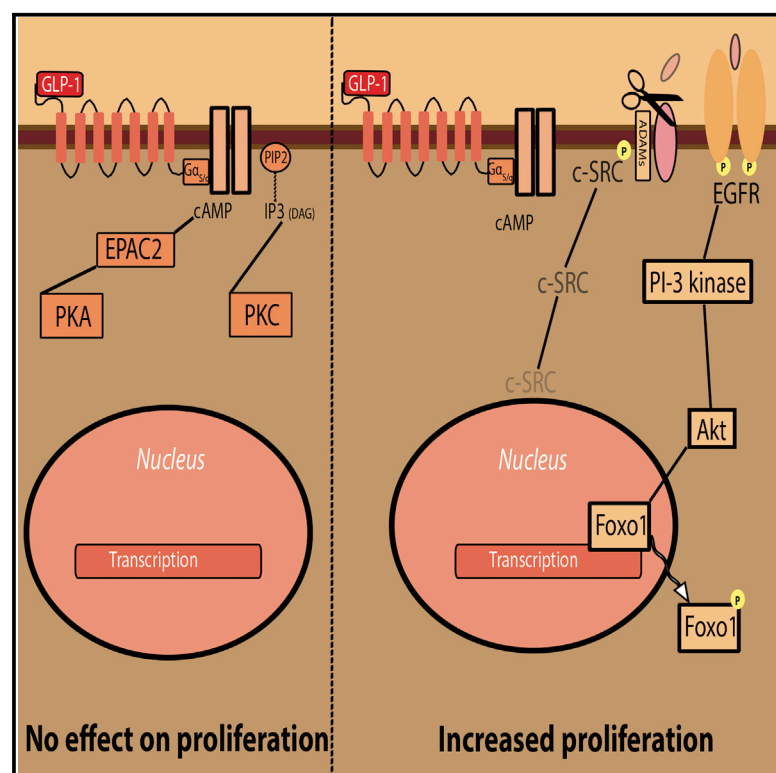
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# Cell Reports

## Glucagon-like Peptide 1 Receptor Signaling in Acinar Cells Causes Growth-Dependent Release of Pancreatic Enzymes

### Graphical Abstract



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### In Brief

Glucagon-like peptide 1 (GLP-1)-based therapies are used to treat type 2 diabetes and obesity. Wewer Albrechtsen et al. detect GLP-1 receptor expression in pancreatic acinar cells and show that its activation leads to mild c-Src-dependent proliferation, increasing constitutive enzyme release. This enzyme increase during GLP-1 treatment does not reflect sub-clinical pancreatitis.

### Highlights

- Glucagon-like peptide 1 does not acutely increase amylase and lipase levels
- Glucagon-like peptide 1 induces mild c-Src-dependent acinar cell proliferation
- This proliferation is associated with an increased constitutive release of enzymes
- Enzyme increase during GLP-1 treatment does not reflect sub-clinical pancreatitis



# Glucagon-like Peptide 1 Receptor Signaling in Acinar Cells Causes Growth-Dependent Release of Pancreatic Enzymes

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## SUMMARY

Incretin-based therapies are widely used for type 2 diabetes and now also for obesity, but they are associated with elevated plasma levels of pancreatic enzymes and perhaps a modestly increased risk of acute pancreatitis. However, little is known about the effects of the incretin hormone glucagon-like peptide 1 (GLP-1) on the exocrine pancreas. Here, we identify GLP-1 receptors on pancreatic acini and analyze the impact of receptor activation in humans, rodents, isolated acini, and cell lines from the exocrine pancreas. GLP-1 did not directly stimulate amylase or lipase release. However, we saw that GLP-1 induces phosphorylation of the epidermal growth factor receptor and activation of Foxo1, resulting in cell growth with concomitant enzyme release. Our work uncovers GLP-1-induced signaling pathways in the exocrine pancreas and suggests that increases in amylase and lipase levels in subjects treated with GLP-1 receptor agonists reflect adaptive growth rather than early-stage pancreatitis.

## INTRODUCTION

Glucagon-like peptide 1 (GLP-1) is a peptide hormone secreted from the gastrointestinal tract in response to nutrient ingestion. GLP-1 potentiates glucose-induced insulin secretion from pancreatic  $\beta$  cells (the incretin effect) and suppresses appetite and food intake (Holst, 2007). GLP-1 receptor agonists (GLP-1RAs) are therefore used for the treatment of type 2 diabetes and obesity (Wewer Albrechtsen et al., 2014; Drucker and Yusta, 2014; Sadry and Drucker, 2013). Their pancreatic safety has been under intense debate (Meier and Nauck, 2014; Butler

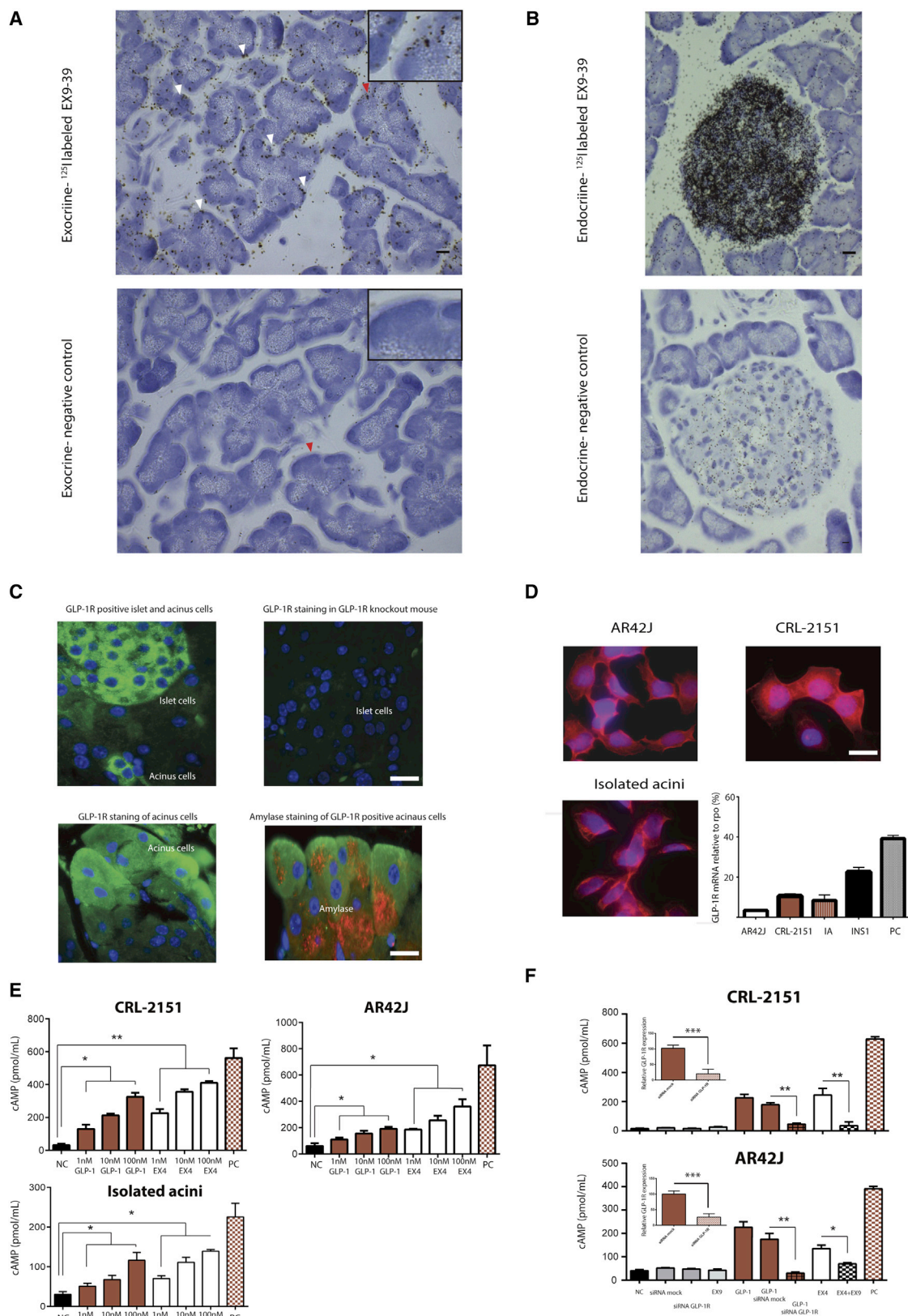
et al., 2013; Elashoff et al., 2011; Egan et al., 2014), since their use has been associated with sporadic cases of acute pancreatitis and generally mildly elevated levels of markers of this disease (amylase and lipase) in animal and clinical studies, although the underlying mechanisms are unknown (Egan et al., 2014). In the present study, we investigated the effects of GLP-1RAs on the release of pancreatic amylase and lipase both in acinar cell lines and in mice, rats, and humans, and as well the molecular mechanism(s) leading to increased levels of pancreatic enzymes.

## RESULTS

### The GLP-1 Receptor Is Expressed on Acinar Cells, and Its Activation Stimulates cAMP Production

Autoradiography with an <sup>125</sup>I-labeled GLP-1R antagonist, exendin 9-39 (EX9-39), revealed specific binding to pancreatic acinar and  $\beta$  cells (Figures 1A and 1B). Binding was blocked by addition of a 1,000-fold excess of unlabeled EX9-39, demonstrating specificity of the binding. Using a well-characterized and validated GLP-1 receptor (GLP-1R) antibody (Pyke et al., 2014), we demonstrated GLP-1R expression in pancreatic acinar cells (positive staining for amylase), which was missing in tissue biopsy specimens from GLP-1R knockout mice (Figure 1C). A commercially available GLP-1R antibody (Figure S1A) showed similar results. Subsequently, we showed expression of GLP-1R in two acinar cell lines and isolated acini, both at the protein level (using the validated GLP-1R antibody) and as messenger transcript, similar to the expression seen in INS1 and GLP-1R transfected HEK293 cells (Figure 1D). Both native GLP-1 and EX4 (a GLP-1RA) stimulated production of cyclic AMP (cAMP) dose dependently in two acinar cell lines and isolated acini (Figure 1E). Blocking the effects of GLP-1R stimulation, either by administering the antagonist EX9-39 or by small interfering RNA (siRNA)-induced knockdown of GLP-1R (~80% and ~74% knockdown for the CRL2151 and the AR42J cell lines,





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respectively, estimated by qPCR; [Figure 1F](#)), resulted in significantly attenuated GLP-1- ( $p < 0.01$ ) and EX4-induced ( $p < 0.01$ ) cAMP levels ([Figure 1F](#)). Appropriate controls, including mock siRNA, GLP-1R siRNA, or EX9-39 alone, showed no significant effect on the levels of cAMP ([Figure 1F](#)).

### Acute Stimulation with Native GLP-1 or a Stable GLP-1RA Stimulates Neither Amylase nor Lipase Levels in Acinar Cell Lines or Mice, Rats, and Humans

The two cell lines (CRL2151 and AR42J) were further characterized by amylase and lipase expression at the transcript and protein levels ([Figures S1B and S1C](#)) and tested functionally by addition of 10 nM CCK-8, which resulted in a robust release of amylase and lipase from both cell lines (5- to 20-fold,  $p < 0.001$ ; [Figure 2A](#)). GLP-1 and EX4, in doses ranging from 1 to 100 nM, neither stimulated nor inhibited amylase ( $p = 0.86$ ) or lipase ( $p = 0.75$ ) activity levels in either of the two acinar cell lines or isolated acinar cells ([Figures 2B and S1D](#)). Administration of the stable GLP-1RA EX4 (10 nM) for 24 hr had no effect on protein levels of either amylase or lipase compared to PBS-treated cells ([Figure 2C](#)). Addition of 1 nM GLP-1 to the arterial perfusate of perfused mouse ( $p = 0.65$ ,  $p = 0.31$ ) and rat ( $p = 0.46$ ,  $p = 0.47$ ) pancreata was also without effect on amylase and lipase release (whereas in both species, insulin secretion was increased; [Figure 2D](#)). In healthy subjects, subcutaneous injection of EX4, GLP-1, or truncated GLP-1 (9-36NH<sub>2</sub>) had no acute effect (120-min period) on plasma levels of amylase ( $p = 0.69$ ,  $p = 0.97$ , and  $p = 0.96$ , respectively) or lipase ( $p = 0.36$ ,  $p = 0.81$ , and  $p = 0.88$ , respectively) compared to placebo ([Figures 2E and 2F](#)).

### 5-Day Treatment with GLP-1RA Increases Levels of Pancreatic Enzymes and Cell Proliferation in Acinar Cell Lines and in Isolated Acini

Administration of either 10 nM GLP-1 or 10 nM EX4 daily for 5 days increased activity levels (~2-fold) of amylase ( $p < 0.001$ ) and lipase ( $p < 0.001$ ) compared to PBS treatment in CRL2151 ([Figures 3A and 3B](#)) and AR42J ([Figure S1E](#)) cell lines, but not in the HEK293 (control) cell line ( $p = 0.78$ ) ([Figure S1F](#)). The stimulatory effects of GLP-1 and EX4 on amylase and lipase levels depended on GLP-1R expression, since the effect was reduced

by knockdown of GLP-1R ([Figures 3A and 3B](#)) (siRNA knockdown efficiency  $\sim 70\% \pm 7\%$  for CRL2151 and  $78\% \pm 5\%$  for AR42J cell lines,  $p < 0.01$ , estimated by qPCR; [Figures 3A and S2A](#)). Of note, daily administration of 10 nM GLP-1 or 10 nM EX4 stimulated proliferation of both acinar cell lines (~2-fold,  $p < 0.001$ ) via a GLP-1R-dependent pathway, which was abolished by both pharmacological (EX9-39) and genetic disruption of GLP-1R (CRL2151 cell line ([Figures 3C and 3D](#)) and AR42J cell line ([Figures S2B and S2C](#); GLP-1R knockdown is depicted in [Figures 3A and S2A](#)). The effect of GLP-1 and EX4 on cell proliferation was similar with each method used to assess proliferation: live-cell imaging (IncuCyte), nucleoside-based analog (bromodeoxyuridine [BrdU]/5-ethynyl-2'-deoxyuridine [EdU]), and antibody staining-based (Ki-67) imaging ([Figures 3C and 3D](#)). GLP-1 or EX4 treatment had no effect on apoptosis in acinar cells (CRL2151) compared to PBS ([Figure S2D](#)). In isolated acini (from 4 mouse pancreases), amylase and lipase activity levels increased after 5 days of EX4 administration concomitantly with an EX-4 induced growth ( $1.8 \pm 0.3$ -fold versus  $1.1 \pm 0.2$ -fold,  $p = 0.03$ , assessed using the IncuCyte technique) ([Figure 3E](#)). The simultaneous administration of EX9-39 blocked both the increases in amylase and lipase activity levels and cell growth. Levels of pancreatic enzymes correlated significantly with cell proliferation (amylase:  $R^2 = 0.88$ ;  $p < 0.0001$  and lipase:  $R^2 = 0.47$ ;  $p = 0.002$ ) ([Figure 3F](#)).

### 7-Day Treatment with a GLP-1RA In Vivo Increases Levels of Pancreatic Enzymes and Acinar Cell Proliferation in Mice

Liraglutide, a long-acting GLP-1RA, significantly stimulated amylase ( $37 \pm 10$  mU/mL versus  $14 \pm 3$  mU/mL,  $p < 0.0001$ ) and lipase ( $9 \pm 2$  mU/mL versus  $3 \pm 2$  mU/mL,  $p < 0.0001$ ) activity levels in plasma of mice compared to PBS treatment ([Figures 4A and 4B](#)). Furthermore, liraglutide also significantly increased pancreas weight ( $8.9 \pm 0.9$  mg/g mouse versus  $7.1 \pm 0.5$  mg/g mouse,  $p = 0.0007$ ; [Figure 4C](#)), although decreases in body weight were not significant ( $-5.7 \pm 1.8$  g versus  $-3.6 \pm 1.2$  g,  $p = 0.20$ ) compared to PBS-treated mice. Liraglutide significantly increased proliferation of acinar cells compared to PBS treatment, both when cell proliferation was estimated by nucleoside-based analog (EdU) ( $3.0\% \pm 1\%$  versus  $0.7\% \pm 0.4\%$

## Figure 1. The GLP-1 Receptor Is Expressed on Acinar Cells, and GLP-1 Receptor Activation Stimulates cAMP Production In Vitro

(A) Microphotographs (hematoxylin stained) of histological sections of a CD1 mouse pancreas using <sup>125</sup>I-labeled EX9-39 without (top) and with (bottom) unlabeled EX9-39 (negative control). The exocrine part of the pancreas is clearly labeled (marked by white arrows; the area pointed to by the red arrow is magnified in the top right field). Most of the isotope is localized to the basal layer of the secretory gland cells, whereas the apical part of the cells seems nearly unlabeled. The lumina of the glands are only partially labeled. Note that most of the extracellular spaces between the exocrine glands are free of iodinated particles.

(B) Microphotographs (H&E stained) of histological sections of a CD1 mouse pancreas using <sup>125</sup>I-labeled EX9-39 without (top) and with (bottom) unlabeled EX9-39 (negative control). The endocrine part (pancreatic islets) of the pancreas is clearly labeled.

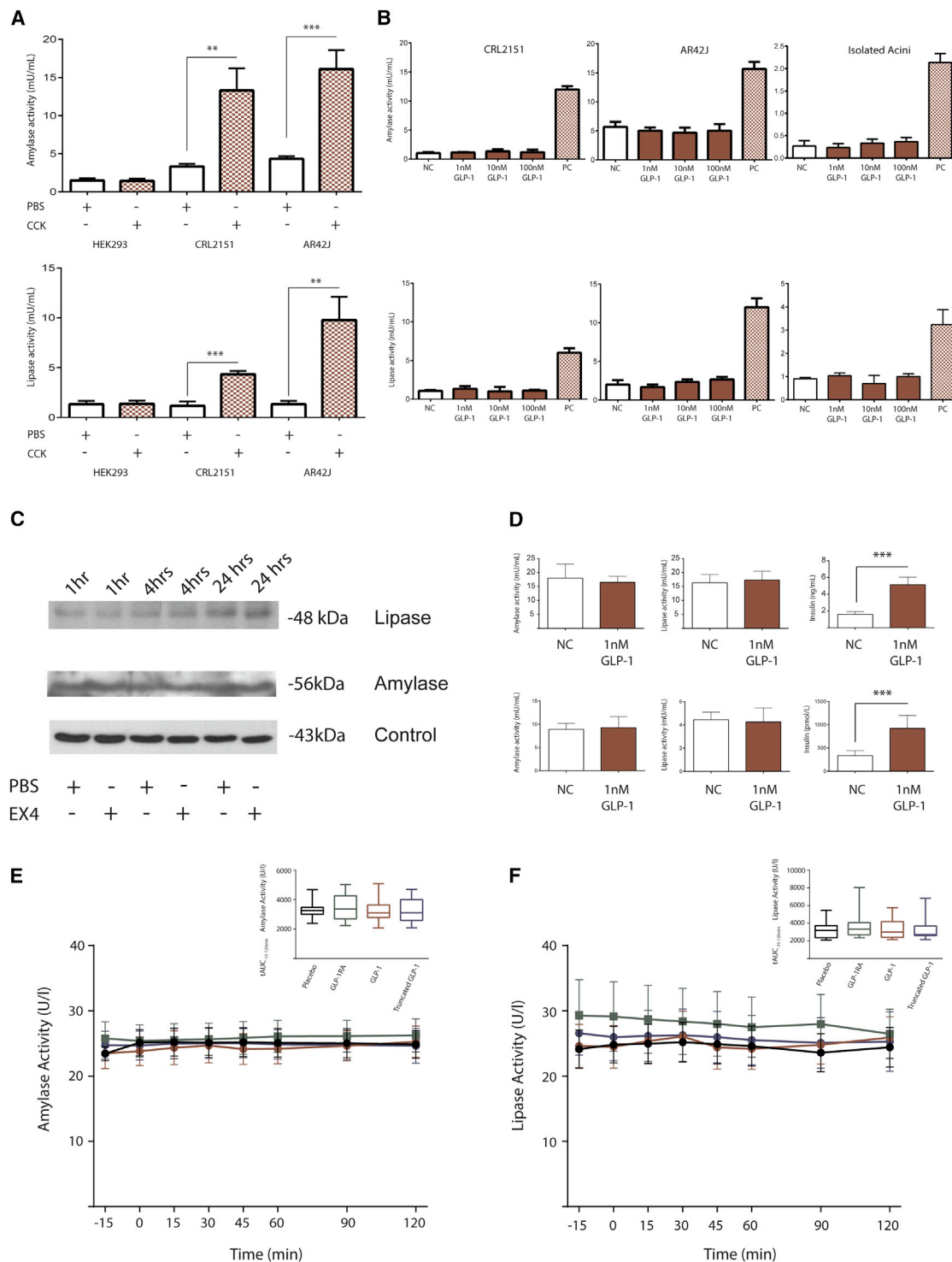
(C) Immunofluorescence using the extensively validated Mab 7F38 GLP-1R antibody of pancreatic tissue biopsy specimens from mice and GLP-1R knockout mice illustrating the expression of GLP-1R on pancreatic acinar cells (also stained for amylase, depicted as red).

(D) Detection of GLP-1R in both acinar cell lines and isolated acini by immunofluorescence (using Mab 7F38) and qPCR relative to the RPLP0 (housekeeping gene).

(E) cAMP production in the two cell lines, CRL2151 and AR42J, and isolated acini after 1 hr of GLP-1 and EX4 stimulation.

(F) cAMP production in CRL2151 and AR42J cells after 1 hr of GLP-1 and EX4 stimulation and simultaneous blockade of GLP-1R by siRNA (relative knockdown of GLP-1R is inserted above each cell line) and EX9-39.

NC, negative control (PBS); PC, positive control. PC refers to overexpression of GLP-1R in (D) and 1  $\mu$ M IBMX, forskolin, and bombesin in (E) and (F). Asterisks represent statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  by one-way ANOVA corrected by a post hoc analysis [Sidak] for multiple testing). Scale bars represent 10  $\mu$ m (A and B) and 12  $\mu$ m (C and D).  $n = 4$ –6 for biological replicates;  $n = 2$ –3 for technical replicates. Values represent mean  $\pm$  SEM.



**Figure 2. Acute Administration of GLP-1 Stimulates Neither Amylase nor Lipase Activity in Acinar Cells, Acinar Cell Lines, or Mice, Rats, and Healthy Subjects**

(A) Amylase activity levels (top) and lipase activity levels (bottom) during PBS and CCK-8 (1 nM) stimulation of CRL2151, AR42J, and HEK293 cells (1 hr).

(B) Amylase activity levels (top) and lipase activity levels (bottom) during GLP-1 stimulation in CRL2151, AR42J, and isolated acinar cells (1 hr).

(C) Western blot profile after 24-hr incubation of CRL2151 cells with EX4 (10 nM) or PBS. From top: lipase, amylase, and control (actin).

(D) Amylase and lipase activity levels in the effluents from perfused pancreases from mice (top) and rats (bottom) during 1 nM GLP-1 administration. Insulin levels were used as a positive control for GLP-1 stimulation.

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EdU positive cells,  $p < 0.0001$ ) (Figure 4D) and by Ki-67 staining ( $3.1\% \pm 0.8\%$  versus  $0.5\% \pm 0.3\%$  Ki-67 positive cells,  $p < 0.0001$ ) (Figure 4E). Notably, the distribution of EdU- and Ki-67 positive cells was independent of their spatial relationship to neighboring islets ( $20 \pm 10$  versus  $3 \pm 5$  EdU/Ki67-positive cells by a  $150 \mu\text{m}$  ratio to insulin-positive cells,  $p = 0.009$ ) (Figure S2E), suggesting that proliferation may not necessarily be related to a GLP-1-stimulated release of insulin. There was no difference in number of EdU-positive islets between the groups (Figure S2F) and no significant changes in total DNA content of the pancreases, but a small and significant ( $p = 0.0045$ ) increase in protein content was found (Figure 4F). Biopsy specimens from the gastrointestinal tract served as positive controls for cell growth (Figure S3A). Plasma levels of insulin were slightly higher after liraglutide compared to PBS treatment, but this did not reach statistical significance (liraglutide-treated mice,  $0.6 \pm 0.1 \text{ ng/mL}$ ; PBS-treated mice,  $0.4 \pm 0.1 \text{ ng/mL}$ ;  $p = 0.12$ ; data not shown).

#### GLP-1-Induced Proliferation of Acinar Cells Is Mediated by Translocation of c-Src and Subsequent Phosphorylation of Epidermal Growth Factor Receptor

None of the three cAMP/IP-3 downstream signaling pathways (protein kinase A [PKA], protein kinase C [PKC] and “exchange protein directly activated by cAMP 2” [Epac2]) were necessary for GLP-1 stimulation of acinar cell proliferation, as specific inhibitors of PKA (KT5720), PKC (calphostin), and Epac2 (HJC 0350) had no effect on GLP-1-induced cell proliferation (Figure 5A).

We therefore tested whether GLP-1-induced cell proliferation was mediated by c-Src/EGFR related pathways (c-Src, epidermal growth factor receptor [EGFR], phosphatidylinositol 3-kinase [PI3K], and Foxo1), as reported for pancreatic  $\beta$  cells (Buteau et al., 2006). Selective inhibitors of c-Src (PP1,  $p < 0.001$ ), EGFR (erlotinib and tyrphostin AG 1478,  $p < 0.01$ ), PI3K (LY294002,  $p < 0.01$ ), and Foxo1 (AS1842856,  $p = 0.079$ ) all attenuated GLP-1RA-induced cell proliferation compared to cells treated with only EX4 or inhibitor alone (Figure 5B). GLP-1-induced phosphorylation of the downstream signaling protein Akt was blocked ( $p < 0.01$ ) by EX9-39; phosphorylation of Akt was also blocked by the inhibition of c-Src, EGFR, and PI3K (Figures S3B and S3C).

To further investigate how GLP-1R leads to c-Src activation, we stained cells using anti-c-Src and c-Src phosphorylation (Tyr416) antibodies during treatment with 50 nM GLP-1 or vehicle. These experiments (Figure 5C) showed that GLP-1R activation mediates phosphorylation of c-Src and subsequently redistributes c-Src from the perinuclear areas to the cell borders. GLP-1 ( $p = 0.009$ ) increased the phosphorylation of the Foxo1 molecule, which subsequently resulted in a redis-

tribution of Foxo1 from the nucleus to the cytoplasm (Figure 5D).

## DISCUSSION

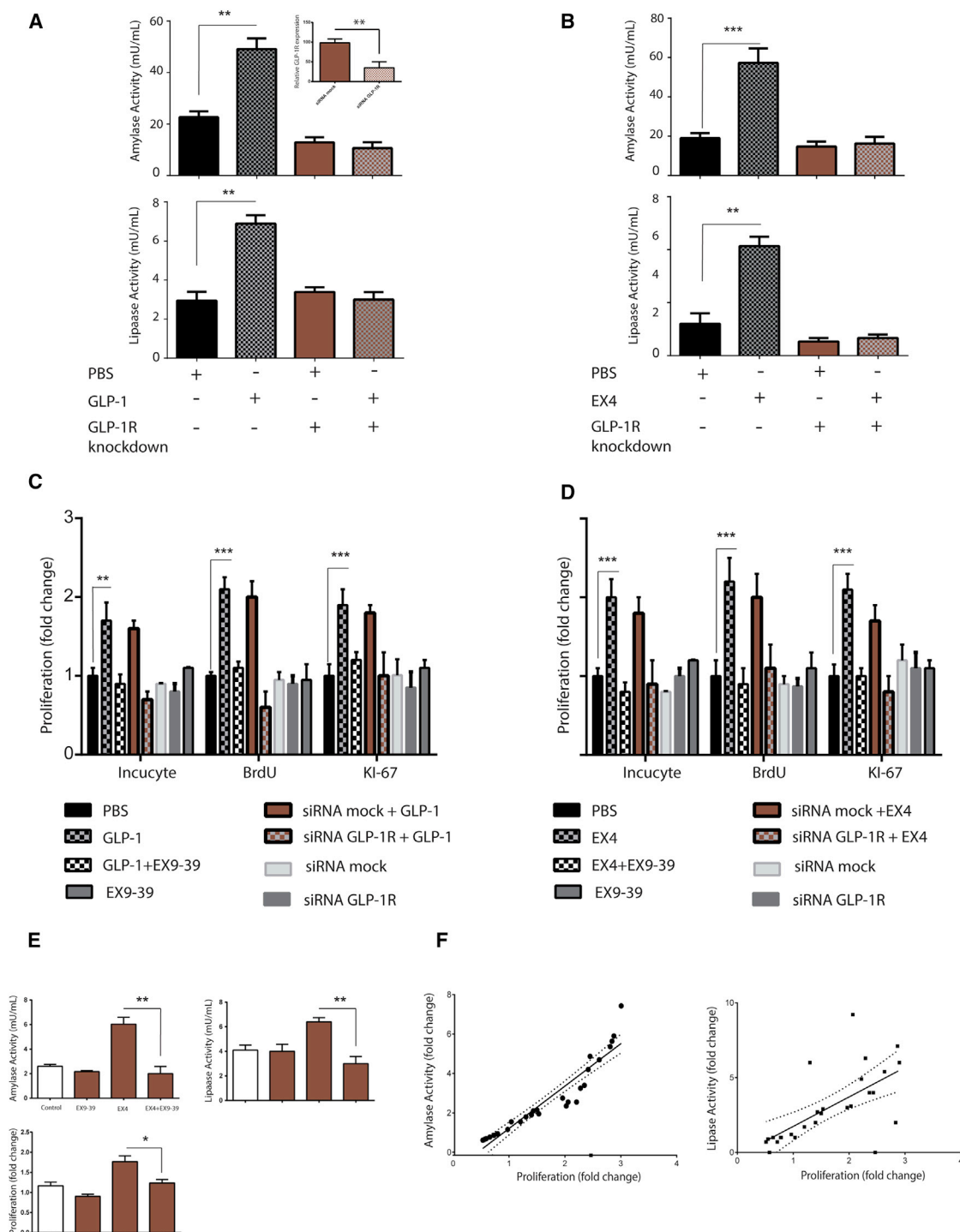
Here, we demonstrate that acute administration of GLP-1 or GLP-1RAs has no effect on the release of the exocrine pancreatic markers lipase and amylase in acinar cell lines, isolated acini, or mice, rats, and healthy human subjects. In contrast, longer-term (4–7 days) treatment of two acinar cell lines, isolated acini, and mice in vivo increased basal secretory rates of amylase and lipase. This was associated with a concomitant proliferation of the acinar cells, which appeared to be coupled to redistribution of c-Src, resulting in phosphorylation of EGFR and activation of its downstream signaling pathways, including the Foxo1 system.

Our data thus provide a mechanistic explanation for the elevated pancreatic enzyme levels in humans treated with GLP-1RA, which differs from the interpretation by others of the increases as being early signs of subclinical pancreatitis (Butler et al., 2013). Our findings are consistent with the recently published joint statement (Egan et al., 2014) by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), who, after considering all available animal (18,000 animals) and clinical data (41,000 participants, including 28,000 exposed to GLP-1 [incretin]-based drugs), concluded that although incretin-based drugs may increase amylase and lipase levels (usually within normal ranges), a coupling to acute pancreatitis could not be established. The absence of a clear relationship is supported by large-scale randomized studies with liraglutide (most recently by the LEADER study) (Pi-Sunyer et al., 2015; Davies et al., 2015; Marso et al., 2016) published after the FDA/EMA report, as well as other large cardiovascular safety outcome studies with GLP-1 receptor agonists (lixisenatide) and DPP-4 inhibitors (which elevate levels of endogenous GLP-1), all showing non-significant changes in acute pancreatitis rates as compared to placebo treatment control groups, albeit with a numerical imbalance in some studies (Scirica et al., 2013; White et al., 2013; Green et al., 2015; Bentley-Lewis et al., 2015). Similar findings were made with liraglutide in a large study in obese nondiabetic individuals, although again, a non-significant imbalance between placebo and liraglutide was noted (Pi-Sunyer et al., 2015). These data do not exclude an association with acute pancreatitis but indicate that this complication must be very rare. However, an explanation for the elevated levels of lipase and amylase could not be provided in these studies.

In our acute studies in humans, we used a therapeutic dose of a stable GLP-1RA (exenatide;  $10 \mu\text{g}$ ), as well as a large subcutaneous dose of native GLP-1 to provide an acute, but therapeutically relevant and relatively long-lasting (given short survival of

(E and F) GLP-1 (red circle), GLP-1 9–36NH<sub>2</sub> (blue circle), and EX4 (green square) administered to eight healthy subjects did not significantly increase the levels of amylase (E) activity ( $p = 0.69$ ,  $p = 0.97$ , and  $p = 0.96$ , respectively) or lipase (F) activity ( $p = 0.36$ ,  $p = 0.81$ , and  $p = 0.88$ , respectively) in plasma compared to placebo (black circle).

Data are shown as mean  $\pm$  SD, and total area under the curve (AUC) (E and F) are shown as boxplots with 25, 50, and 75 percentiles and whiskers (minimum and maximum value). NC, negative control (PBS); PC, positive control (100 nM CCK-8). Asterisks represent statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$  by one-way ANOVA corrected by a post hoc analysis [Sidak] for multiple testing).  $n = 4$ –8 for biological replicates;  $n = 2$ –3 for technical replicates. Values represent mean  $\pm$  SEM.



**Figure 3. GLP-1RA Increases Levels of Amylase and Lipase Concomitantly with Proliferation in CRL2151, AR42J, and Primary Acinar Cells** (A and B) Amylase and lipase levels in incubation media after daily administration of 10 nM GLP-1 (A) or 10 nM EX4 (B) for 5 days to CRL2151 cells with or without GLP-1R siRNA. Relative GLP-1R expression is inserted above in (A).

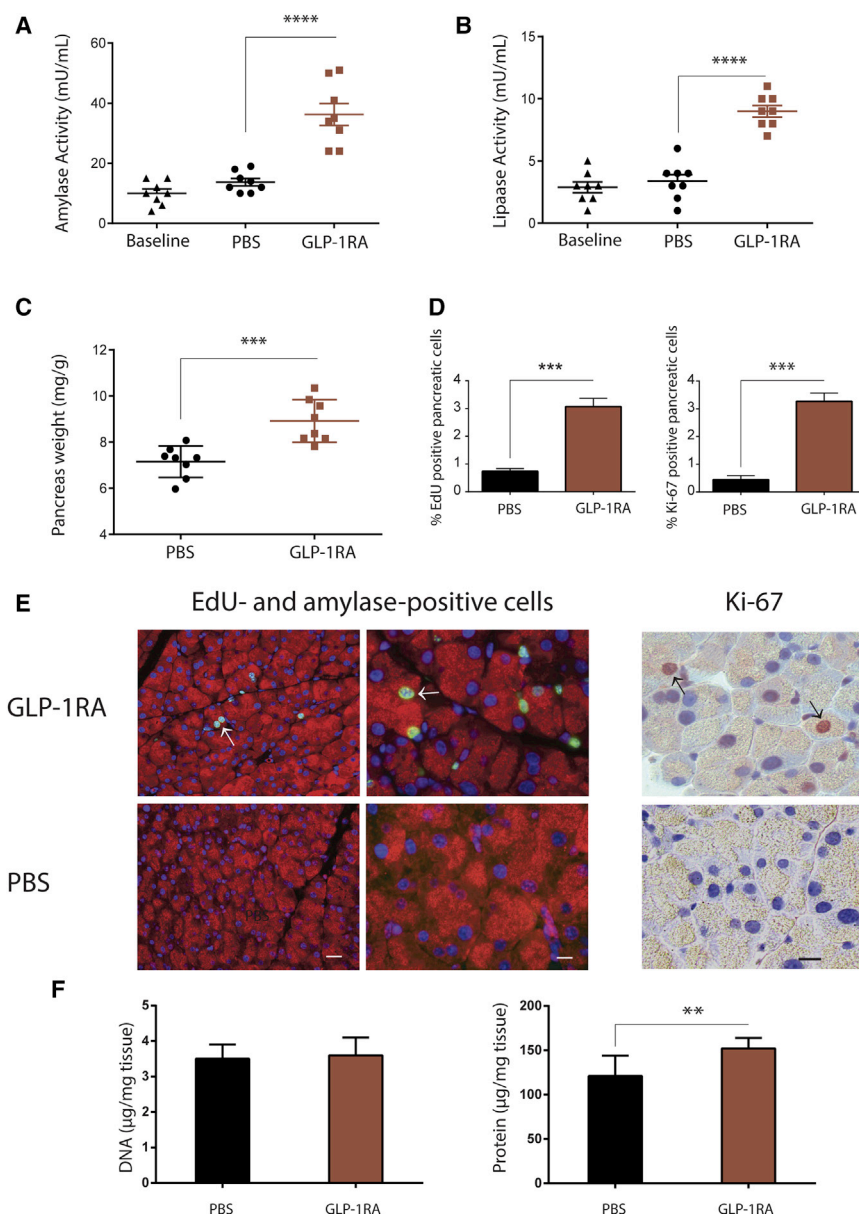
(C and D) Cell proliferation estimated by IncuCyteZOOM, BrdU, or KI-67 after 5-day administration of 10 nM GLP-1 (C) or 10 nM EX4 (D) to CRL2151 cells with or without EX9-39 or GLP-1R siRNA. Administration of EX9 or siRNA alone did not affect cell growth compared to PBS-treated cells.

(E) Amylase and lipase activity levels increased after 5-day daily EX4 administration with or without EX9-39 in primary acinar cells.

(F) Correlations between amylase (left) or lipase levels (right) with the proliferation index in CRL-2151.

Asterisks represent statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  by one-way ANOVA corrected by a post hoc analysis [Sidak] for multiple testing.  $n = 6$ –8 for biological replicates;  $n = 2$ –3 for technical replicates. Values represent mean  $\pm$  SEM.





**Figure 4. Liraglutide Increases Levels of Amylase and Lipase Concomitantly with Proliferation in Mice**

(A and B) Plasma levels of amylase (A) and lipase activity (B) increase during 7-day treatment with the GLP-1RA liraglutide in mice.

(C–E) Pancreas weight (C) and number of EdU- and Ki-67-positive acinar cells (D) based on staining of the corresponding pancreas tissue (E) increased significantly in liraglutide-treated mice ( $n = 8$ ) compared to PBS-treated mice ( $n = 8$ ). In (E), cells were also stained for amylase protein (red).

(F) Protein content (right) and DNA content (left) from pancreas biopsy specimens from PBS-treated and liraglutide-treated mice.

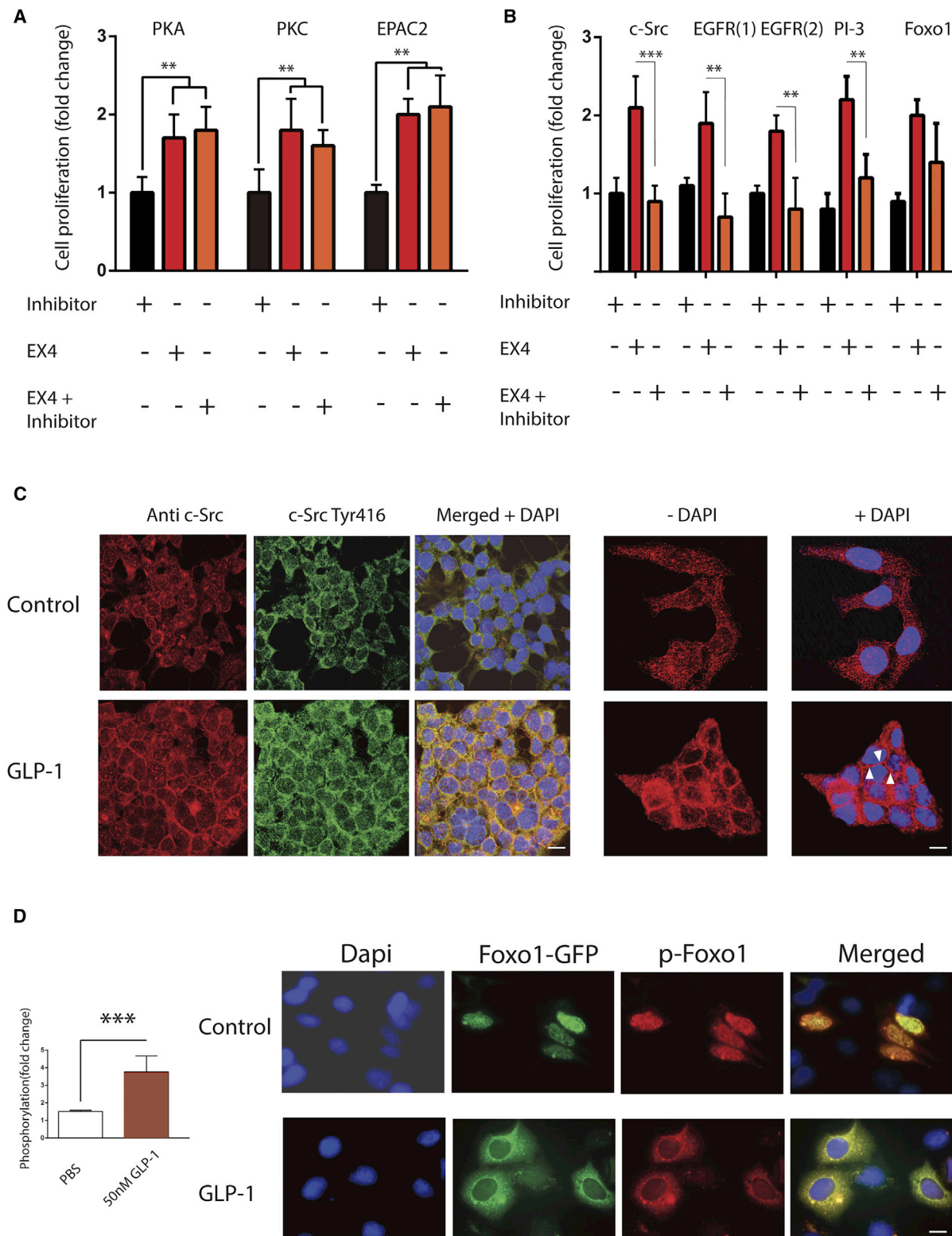
Asterisks represent statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$  by one-way ANOVA corrected by a post hoc analysis [Sidak] for multiple testing).  $n = 8$  for biological replicates;  $n = 2$ – $3$  for technical replicates. Scale bars represent 40  $\mu\text{m}$  (E, left), 20  $\mu\text{m}$  (E, middle), and 15  $\mu\text{m}$  (E, right). Values represent mean  $\pm$  SEM.

but not GLP-1R-deleted, mice (Hou et al., 2016). In agreement with the original studies by Raufmann and Eng (Singh et al., 1994), there was also an increase in cAMP, but the latter authors were unable to demonstrate amylase release, and in studies using isolated perfused pig pancreases, which secrete pancreatic juice and enzymes at normal in vivo rates, GLP-1 had no effects on enzyme secretion (Holst et al., 1993), in agreement with the observations presented here. Moreover, the apical release of zymogen granules from acinar cells is normally not associated with increases in plasma levels of the enzymes, the main focus of the present studies, in spite of increases in stimulatory hormones and in plasma GLP-1. A very recent research letter described initial decreases, lasting

intact GLP-1 in the circulation) and robust elevation of the plasma concentrations. Moreover, since the predominant circulating form of endogenous GLP-1 is the N-terminally truncated product of DPP-4 action, GLP-1 9–36 NH<sub>2</sub> (Deacon et al., 1995), which may share some effects with GLP-1 (Ban et al., 2008), we also examined the effects of this peptide at relevant doses. The lack of acute effect on levels of pancreatic enzymes is in agreement with data showing no effect of meal-induced increases in endogenous GLP-1 in patients with type 2 diabetes (Sonnen et al., 2015). Similarly, there were no acute effects of GLP-1 or GLP-1RA on pancreatic enzyme secretion in two acinar cell lines, isolated acini, and perfused pancreases from mice and rats. Hou et al. recently reported increased amylase activity in the incubation medium of mouse acini isolated from wild-type,

150 min, with extremely small (3–4 U/L) and late (2.5 hr after a meal) increases in amylase (and no effect on lipase) in subjects with type 2 diabetes after infusion of exendin 4, which, considering the powerful effects on upper gastrointestinal motility and secretion, would be difficult to interpret in relation to pancreatic secretion or release (Smits et al., 2016).

In contrast, longer-term (4–6 days) treatment of mice with GLP-1RA was associated with increased basal levels of amylase and lipase, which occurred in parallel with increases in the mitosis rates of acinar cells. These results regarding GLP-1-induced proliferation are consistent with other data obtained in mice (Ellenbroek et al., 2013), rats (Perfetti et al., 2000; Gier et al., 2012; Nachnani et al., 2010), and monkeys (Nyborg et al., 2012). A more recent study suggested that GLP-1R



**Figure 5. GLP-1 Effect on Cell Proliferation Is Mediated by EGFR Signaling through Phosphatidylinositol 3-Kinase, Akt Phosphorylation, and the Foxo1 System**

(A) Inhibiting cAMP-related pathways had no effect on EX4-induced cell proliferation of the CRL2151 cell line.

(B) Specific inhibitors of c-Src (20 nM), EGFR (1 mg/mL), PI3K (1  $\mu$ M), and foxo1 (50 nM) all resulted in attenuated EX4-induced cell proliferation.

(legend continued on next page)

activation results in increased protein synthesis (and therefore increased pancreatic mass) rather than cell proliferation (Koehler et al., 2015). We could clearly show in vitro not only proliferation but also its dependence on GLP-1R expression, suggesting a direct effect of GLP-1 on acinar cells, as opposed to indirect pathways involving, for instance, increased insulin secretion from pancreatic  $\beta$  cells or, for the in vivo experiments, non-pancreatic factors. Finally, we also provide mechanistic insight on how GLP-1 and GLP-1RA might affect the acinar cells. Classical GLP-1R signaling ( $G_{\alpha s}$ ) involves production of cAMP with subsequent activation of PKA and the Epac family. However, there is evidence that GLP-1R signaling in  $\beta$  cells may also involve an EGFR-dependent activation of the Foxo1 system, supporting  $\beta$  cell rejuvenation (Carter and Brunet, 2007; Hall et al., 2000). Since little is known about GLP-1R signaling in exocrine acinar cells, we investigated whether GLP-1-induced proliferation was mediated through cAMP-mediated mechanisms and found, to our surprise, that this was not the case. Instead, we identified an intracellular mechanism for GLP-1R signaling involving c-Src that eventually leads to activation of EGFR. Indeed, c-Src and subsequent EGFR phosphorylation were essential for GLP-1R signaling in acinar cells.

Collectively, our data demonstrate that GLP-1-induced increases in amylase and lipase are dependent on c-Src signaling and acinar cell growth. We included two acinar cell lines (CRL-2151 and AR42J, the latter differentiated with steroids toward an acinar phenotype; Logsdon et al., 1985) and isolated acini (from mice) and demonstrated that GLP-1R activation eventually leads to cell proliferation. The biological relevance of the in vitro results was substantiated by the observations made after daily injection for 7 days with the GLP-1RA liraglutide, which significantly stimulated plasma levels of amylase and lipase in mice concomitantly with proliferation of acinar (amylase-positive) cells. This suggests that GLP-1 does not stimulate the release of pancreatic enzymes per se but increases circulating levels of these enzymes due to a growth-dependent increase of their basal activities. The mild increase in proliferation appears to be an acute adaptive response, which is fully in line with studies showing no adverse histological changes or increases in pancreatic cancer (Ueberberg et al., 2016; Azoulay et al., 2016).

In summary, our findings suggest that the elevated amylase and lipase levels in GLP-1-treated subjects reflect adaptive growth of pancreatic acinar cells rather than subclinical pancreatitis.

## EXPERIMENTAL PROCEDURES

### Human Experiments

Eight normoglycemic healthy subjects (three females and five males; age, 24  $\pm$  3 years; weight, 71  $\pm$  11 kg; BMI, 23  $\pm$  1) were included in this double-blinded

crossover study. Subcutaneous injections of saline, GLP-1 7-36NH<sub>2</sub> (1.5 nmol/kg), GLP-1 9-36NH<sub>2</sub> (1.5 nmol/kg), or exendin-4 (EX-4; 10  $\mu$ g) were given at time zero on four different days with a minimum of 2-day intervals within a total time period of 3 months, and blood samples were obtained at –15, 0, 15, 30, 45, 60, 90, and 120 min. The study was conducted according to the latest revision of the Helsinki Declaration and approved by the Scientific-Ethical Committee of the Capital Region of Denmark and by the Danish Data Protection Agency (application SJ-497). Written informed consent was received from participants prior to inclusion in the study.

### Animal Experiments

All animal experiments were approved by the Danish National Committee for Animal Studies, Ministry of Justice (2013–15–2934–00833) and were conducted in accordance with the EU Directive 2010/63/EU and guidelines of Danish legislation governing animal experimentation (1987) and the NIH (publication number 85-23) as previously described (Kuhre et al., 2014; Wewer Albrechtsen et al., 2016).

### In Vivo Animal Experiments

Female C57BL/6J mice (~20 g; Janvier Labs) were housed eight per cage under standard conditions for at least 2 weeks before experiments. Mice were divided into weight-matched groups (each 22  $\pm$  1 g) receiving two daily (morning and evening) subcutaneous (s.c.) injections of liraglutide (Novo Nordisk A/S) (0.2 mg/kg, 100  $\mu$ L) or PBS (100  $\mu$ L). Both groups also received two daily s.c. injections of EdU (catalog number BCK488-IV-IM-M, Sigma-Aldrich; 50 mg/kg, 200  $\mu$ L) to assess cell proliferation. Seven days later, the mice were anesthetized with an s.c. injection of ketamine (catalog number 511485, Ketaminol, Intervet, Merck, dose 100 mg/kg) and xylazine (catalog number 148999 Rompun vet, Bayer AG, dose 10 mg/kg). The abdomen was opened and a needle inserted into the caval vein. A maximal blood sample was collected and transferred to an EDTA-coated tube (catalog number 367841, Becton, Dickinson and Company). The samples were centrifuged (1,650  $\times$  g, 4°C, for 10 min) and plasma was immediately frozen at –20°C. Pancreas tissue was harvested and fixed in 10% (w/v) formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C. The tissue was then dehydrated in ethanol and xylol and finally embedded in paraffin wax. 3- $\mu$ m-thick paraffin sections were cut for immunohistochemistry and placed on Superfrost Plus glass slides. Immediately after collection of the pancreatic tissue, the animals were killed by exsanguination.

### Cell Culture

HEK293-VnR cells were generated from HEK293 cells by stable overexpression of  $\alpha V\beta 3$  integrin (Sanjay et al., 2001). The CRL-2151 (266-6) acinar pancreatic cell line, derived from a mouse tumor induced with an elastase I/SV-40 T antigen fusion gene (Ornitz et al., 1985), and the AR42J rat pancreatic tumor cell line were both from ATCC. The AR42J cell line was differentiated toward an acinar-like phenotype as described previously (Logsdon et al., 1985). INS-1E cells were kindly provided by Professor Jens Højris Nielsen (University of Copenhagen, Denmark). Isolation of murine acini was performed using a well-established method described previously (Gout et al., 2013). In brief, four murine pancreases were dissected and processed using the protocols kindly provided by Gout et al., and the isolated acini were seeded in six-well culture dishes (2 mL per well) and cultured at 37°C under 5% (v/v) CO<sub>2</sub> atmosphere. Cells were grown in DMEM (catalog number 31966-021, Gibco) containing 5,000 U/mL penicillin/streptomycin (catalog number 15140-122, Gibco) and 10% fetal bovine serum (FBS) (catalog number Sv3016003,

(C) GLP-1 phosphorylates and subsequently redistributes c-Src from perinuclear areas to cell borders in CRL-2151 cells. Top: control (PBS). Bottom: GLP-1 treated (48 hr). Left: anti-c-Src antibody (red) and anti-c-Src (green: phosphorylated Tyr416) antibody. Right: cells stained for c-Src with larger magnification and merged with DAPI. White arrowheads point to localization of the c-Src of the cell membrane. The left panels show that GLP-1 induces phosphorylation of the c-Src molecule, and this subsequently leads to redistribution of the c-Src molecule to the cell membrane as shown in the right panels.

(D) GLP-1 (incubation for 48 hr) increased phosphorylation of Foxo1 and increased translocation of foxo1 out of the nuclei. The IncuCyteZOOM was used to assess cell proliferation in the above mentioned panels. Asterisks represent statistical significance (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, and \*\*\*\* $p$  < 0.0001 by one-way ANOVA corrected by a post hoc analysis [Sidak] for multiple testing).  $n$  = 5–7 for biological replicates;  $n$  = 2–3 for technical replicates. Scale bars, 12  $\mu$ m. Values represent mean  $\pm$  SEM.



Thermo Fisher Scientific). The cells were seeded in 24-well plates (Nunc, Thermo Fisher Scientific) at a cell density of  $4 \times 10^4$  per well.

### Ex Vivo Experiments

Isolated mouse and rat pancreases were perfused as described in [Supplemental Experimental Procedures](#).

### In Vitro Experiments

For acute stimulation protocols, we used ~80% confluent cells from different batch numbers ( $n = 3$ ). Cells were stimulated with GLP-1, EX4, and EX9-39 (concentration range 1–1,000 nM) for up to 1 hr. In pilot studies, we included stimulation periods of 5, 10, 30, and 60 min. For longer-term stimulation, similar numbers of cells were placed in each well of 12-well plates; every second hour (up to 120 hr), the cells were automatically counted using IncuCyte Zoom equipment (Essen BioScience). In addition, BrdU (Click IT) was administered daily together with stimulants (GLP-1, EX4, EX9-39, or enzyme inhibitors). After the end of the stimulation period, cell media were obtained and centrifuged ( $1,500 \times g$ ,  $4^\circ\text{C}$ , 5 min) to remove any cells or debris and kept at  $-80^\circ\text{C}$  until analysis.

### Antibodies and Proteins

GLP-1R antibody (Monoclonal antibody [Mab] 7F38) was deposited to the Developmental Studies Hybridoma Bank (DSHB) by Knudsen, L.B. (DSHB Hybridoma Product Mab 7F38) and used for [Figure 1](#). The peptides GLP-1 7-36NH<sub>2</sub> (catalog number H-6795), GLP-1 9-36NH<sub>2</sub> (catalog number H-4012), exendin 9-39 (EX9-39; catalog number H-8740), exendin-4 (EX4; catalog number H-3864), and sulfated cholecystokinin octapeptide (CCK-8; catalog number H-2080) were all from Bachem. Other reagents are listed in [Supplemental Experimental Procedures](#).

### Proliferation Assay

IncuCyte ZOOM (Essen BioScience) was used to analyze and estimate the proliferation grade of the cultured cells as previously described ([Stewart et al., 2015](#); [Craveiro et al., 2013](#); [Dombrowsky et al., 2015](#)). For a detailed description, see [Supplemental Experimental Procedures](#). IncuCyte ZOOM phase images were taken automatically every second hour, which made it possible to monitor cellular growth, behavior, and morphology by live-cell imaging (label-free confluence). In addition, the machine delivers an effective kinetic readout, i.e., graphs showing proliferation for individual cell culture wells. Raw data (phase object confluence and percentage) were used for illustrations without normalization. Proliferation reflects the percentage increase of the phase object confluence (percent). In parallel, two additional proliferation assays were performed using the Click-IT EdU and BrdU imaging kits (catalog number C10337, Invitrogen) according to the manufacturer's protocol. To estimate apoptosis, we used a colorimetric TUNEL assay from Trevigen (catalog number 4822-96-K). For the in vivo study, in addition to the EdU and staining by Ki67 (see below), we also assessed protein and DNA content. In short, pancreas tissues (remaining pieces) were homogenized in a lysis buffer as described by others ([Koehler et al., 2015](#)) and sonicated for 1 min. A standard biocinchoninic acid (BCA) protein assay from Sigma (catalog number BCA-1) and a Quant-iT dsDNA assay from Thermo Fisher Scientific (catalog number Q33120) were used.

### qPCR and Small Interfering RNAs

For a detailed description, see [Supplemental Experimental Procedures](#).

### Immunohistochemistry, Immunofluorescence, and Autoradiography

Pancreatic acinar cell proliferation estimations were performed by acquiring EdU/amylose/DAPI images of each section from the 16 mice. Autoradiography was performed using <sup>125</sup>I-labeled EX9-39 as described previously ([Jensen et al., 2015](#)).

### Western Blot Analysis

For a detailed description, see [Supplemental Experimental Procedures](#).

### Biochemical Measurements

Pancreatic enzyme activities, expressed as mU (mmol/min/mL), were measured (blinded) using commercial colorimetric assays (amylase [ab102523] and lipase

[ab102524], with lower limits of detection of 0.2 and 0.1 mU, respectively; both from Abcam). For amylase and lipase activity in plasma samples, pancreatic  $\alpha$ -amylase and triacylglycerol lipase (pancreatic lipase) were measured on a Cobas 8000 c502 platform (Roche Diagnostics GmbH) using Roche  $\alpha$  amylase EPS pancreatic reagents or Roche Lipase colorimetric assay, including Calibrator f.a.s. For a detailed description, see [Supplemental Experimental Procedures](#).

### Statistics

One-way ANOVA, corrected by a post hoc analysis (Sidak) for multiple testing, was used for testing differences between more than two groups of data.  $p < 0.05$  was considered significant. For a detailed description, see [Supplemental Experimental Procedures](#).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.11.051>.

### AUTHOR CONTRIBUTIONS

N.J.W.A., R.A., L.B., B.S., R.E.K., S.S.P., C.B.C., E.P.J., C.J., L.H., C.F.D., B.H., and J.J.H. contributed substantially to the concept and design of the study; N.J.W.A., R.A., L.B., B.S., R.E.K., S.S.P., C.B.C., E.P.J., C.J., L.H., C.F.D., and B.H. contributed substantially to the analysis and interpretation of the data; N.J.W.A. and J.J.H. drafted the manuscript; and R.A., L.B., B.S., R.E.K., S.S.P., C.B.C., E.P.J., C.J., L.H., C.F.D., and B.H. critically revised the manuscript for important intellectual content. All authors provided approval of the final version to be published. J.J.H. is responsible for the integrity of the work as a whole.

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